Award Number: DAMD17-02-1-0396

TITLE: Differential Control of ErbB2 Surface Expression in Breast Cancer Cells by an Alternatively Spliced Form of ERBIN

PRINCIPAL INVESTIGATOR: Jeremy W. Peck

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

REPORT DATE: May 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Annual Summary (1 May 2002 - 30 Apr 2003)

4. TITLE AND SUBTITLE
Differential Control of ErbB2 Surface Expression in Breast Cancer Cells by an Alternatively Spliced Form of ERBIN

5. FUNDING NUMBERS
DAMD17-02-1-0396

6. AUTHOR(S)
Jeremy W. Peck

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20007
E-Mail: Jeremy_peck@hotmail.com

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Receptor internalization following ligand stimulation is an important way of attenuating downstream signaling. One such family of proteins involved in this process are the Vps proteins. Vps proteins are a family of proteins first identified in Yeast that are required for proper vacuolar protein sorting (hence the name Vps). Yeast Snf7p is a small coiled-coiled protein involved in multivesicular body (MVB) function. Genomic and proteonomic studies indicate yeast Snf7p also interacts with Bro1-containing proteins, Bro1p and Rim20p, involved in MVB function and pH signal transduction, respectively. Here we report the identification of Snf7-1, one of a family of at least three human homologs of yeast Snf7p. Using affinity-capture experiments, we show that human Snf7-1 interacts with AIP1, a mammalian Bro1p-containing protein involved in apoptosis and cellular vacuolization. Snf7-1 did not, however, interact with another human Bro1-containing molecule, Rhophilin-2. Additional domain mapping using affinity-capture experiments revealed that the N-terminus of AIP1 was necessary and sufficient for interacting with Snf7-1. These results suggest the possibility that the Snf7-1-AIP1 interaction plays a role in mammalian MVB function.

14. SUBJECT TERMS

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Results.....4

Key Research Accomplishments.....8

Reportable Outcomes.....8

Conclusions.....8

References.....9

Appendices.....

I. INTRODUCTION

Although the original focus of the project was the relationship of Erbin to Erb2/Her2 signaling, the Erbin story has become not relevant to RbB2 signaling. The reason for this is that recent studies suggest the Erbin-ErbB2 interaction is non-physiological and that Erbin likely regulates p120-catenin
which shows ~1000-fold higher binding activity than that with ErbB2. In
light of this we have changed the focus of the project to study human Vps32 and Vps20 proteins, putative homologues of yeast proteins, which we hypothesize control ErbB2 receptor trafficking.

Ligand-induced down-regulation of mitogenic receptors, which involves receptor endocytosis via clathrin-coated vesicles, is crucial in regulating mitogenic signals (1). Sorting of endocytosed receptors is a complex, multi-step process that includes their relocalization to the internal vesicles of late endosomes or multivesicular bodies (MVB), from which they are then either recycled to the surface or targeted to lysosomes for degradation. Thus, degradation by lysosomes plays an important role in down-regulating many activated cell-surface receptors (1). One family of proteins, the class E vacuolar protein-sorting (*vps*) proteins, is important for endosomal sorting as shown by *vps* mutants that fail to transport newly synthesized hydrolases efficiently to the vacuole in yeast (2). More recently, yeast studies identified three protein complexes termed ESCRT-I, -II and-III (endosomal sorting complex required for transport) involved in different stages of sorting ubiquitylated endosomal membrane proteins into the MVB pathway (3,4). ESCRT I is involved in binding the ubiquitinylated cargo and activates ESCRT-II, which then assembles the ESCRT III complex needed for membrane association and sorting of the MVB cargo (3,4)

The yeast ESCRT-III complex is composed of at least four proteins, Snf7p, Vps2p, Vps20p and Vps24p (4). The Vps20p-Snf7p subcomplex is associated with the MVB membrane and may have an important role in sorting and/or determining the composition of the MVB cargo. Snf7p is a relatively small, charged, coiled-coiled protein that was originally identified genetically in a screen for mutants unable to sense glucose concentration changes (5,6). Snf7p mutants also block endosome to vacuole trafficking, resulting in structures resembling late endosomes (6). Consistent with a role for Snf7p in MVB structure and/or function, a proteonomic yeast study showed that Snf7p interacted with Vps4p as well as Bro1p (otherwise known as Npi3p and Vps31) (7). Bro1p, a scaffold protein containing an N-terminal Bro1 domain, is also involved in the MVB sorting pathway (8). Bro1p functions in the ubiquitin-dependent destruction of activated cell surface receptors (9) and has been identified as a class E VPS protein (8). A large-scale two-hybrid screen recently revealed that Snf7p also interacts with another Bro1-containing yeast protein, Rim20p (10). Rim20p is a scaffold protein required for the proteolysis of the Rim101 transcription factor, which is involved in mediating pH transcriptional responses to environmental pH changes in *Sacharomyces cerevisiae* (11).

Rim20p homologs, each with a highly conserved Bro1 domain, exist in a number of different organisms including fungus (12,13), amphibians (14), mice (15,16) and humans (13,17). The Rim20p homologue in fungus, *Aspergillus nidulans,* PalA protein, is also

involved in the assembly of a protease cleavage complex required for the processing of transcription factors involved in alkaline pH adaptation (12,13). The mouse Rim20p homolog, AIP1 (also known as ALIX), was originally identified in a yeast two-hybrid screen as a protein interacting with ALG2, a protein involved in apoptosis (15,16). In addition to an N-terminal Bro1 domain, the C-terminal 150 amino acids of AIP1 is proline rich and can interact with the SH3 domains of several other proteins including SETA/RUK (18,19) and endophilins (20). Overexpression of the AIP1 C-terminus has a variety of biological affects including inhibiting cell death (15,18) and inducing vacuolization (20). Finally, the function of the Bro1 domain in AIP1 and other Bro1-containing molecules is not known.

Here we have identified one of three human homologs of yeast Snf7p. Snf7-1 is 222 amino acids long and is 38% identical to yeast Snf7p. After testing Using affinity-capture experiments, we show that the N-terminus of AIP1 was necessary and sufficient for interacting with human Snf7-1. These results suggest the possibility that the interaction of human Snf7-1 is likely to be biologically significant and may be involved in regulating MVB function.

II. RESULTS

Identification of Human Homologies of Yeast Snf7p/Vps32 and Vps20

Using the human EST and NR databases we searched for human proteins homologous to the yeast Snf7p. One of the cDNAs identified and sequence confirmed was found to encode a protein of 222 amino acid residues (Fig. 1). Analysis of the nucleotide sequences of the Snf7-1 clone confirmed that it contained a Kozak's consensus translation initiation sequence 5' to the methionine start codon and an in frame stop codon (data not shown). The predicted protein encoded by Snf7-1 is of similar size to yeast Snf7p and is 38% identical at the amino acid level over the entire molecule (Fig. 1). Comparison of Snf7-1 with other related human proteins revealed at least two additional related moloecules, Snf7-2 and Snf7-3. Snf7-2 and Snf7-3 were full length cDNAs and encoded proteins of similar size (Fig. 1). All three human Snf7 homologs, like yeast Snf7p, have amino acid sequences that are likely to fold into coil-coiled secondary structures within these proteins (data not shown).

Examination of the EST database revealed that Snf7-1 is ubiquitously expressed (data not shown) suggesting a potential general role in cellular function. Finally, additional database searches revealed additional Snf7 homologies in fungus, fly, worms and plants.

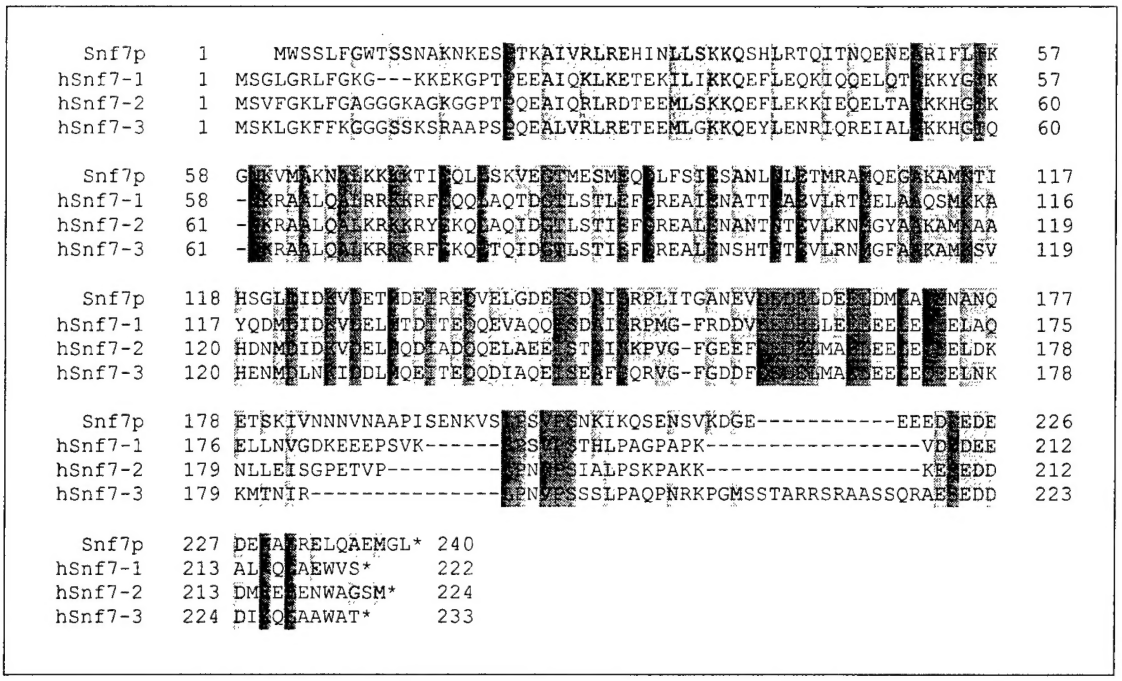

Figure 1 Identification of Human Homologs of Yeast Snf7p.
Using BLASTp of human sequences within the NCBI protein database was used to find the homologous sequences. Protein sequence analyses were performed using the AlignX function of the VectorNTI Suite version 6.0.5 software (InforMax Inc.). A. Human amino acids identical or conserved with Snf7p from *Sacchromyces cerevisiae* are shown in dark and light shading, respectively. Three human Snf7p homologs were identified.

hSnf7-1 Interacts with AIP1, but not Vps20, Vps4-A or PalBH
In yeast, Snf7p interacts with multiple proteins including Vps4p, Rim13p (10), a calpain protease and two different yeast Bro1 domain-containing proteins: Bro1 and Rim20 (11,13). Additionally, other studies show that Vps4p interacts with Bro1 and Rim20 (21). In light of these results and to determine if mammalian counterparts of these yeast proteins interact in a similar fashion, we used an affinity capture approach to examine protein-protein interactions. In these experiments, we produced epitope-tagged proteins from expression vectors for : hSnf7-1; hVps20; AIP1, a human Bro1 domain-containing homolog of yeast Rim20p and Bro1 (15,16); Vps4-A, a rat homolog of Vps4p (22) ; and PalBH (23), a mammalian calpain protease homolog of Rim13p. Western blot analysis of Cos1 cells transfected with these different mammalian expression vectors revealed that these expressed proteins migrated as expected for their predicted molecular weights (See Fig. 2). In our first set of experiments, we tested the ability of recombinant GST-hSnf7-1, left immobilized to glutathione beads, to bind epitope-tagged proteins in Cos1 cell extracts. In these experiments, we found that AIP1 interacted strongly with GST-hSnf7-1 but not with GST (Fig. 2). While hVps20, Vps4-A or PalBH were also expressed at relatively high levels, these proteins did not interact with the immobilized GST-hSnf7-1 (Fig. 2). Experiments using GST-Vps4-A and GST-hVps20 bound to glutathione beads also did not detect interactions with either hSnf7-1 or AIP1 (data not shown). These

results suggest that only one of the known homologous yeast interactions, the interaction of hSnf7-1 with AIP1, could be confirmed to occur in mammalian cells using our GST-affinity capture approach.

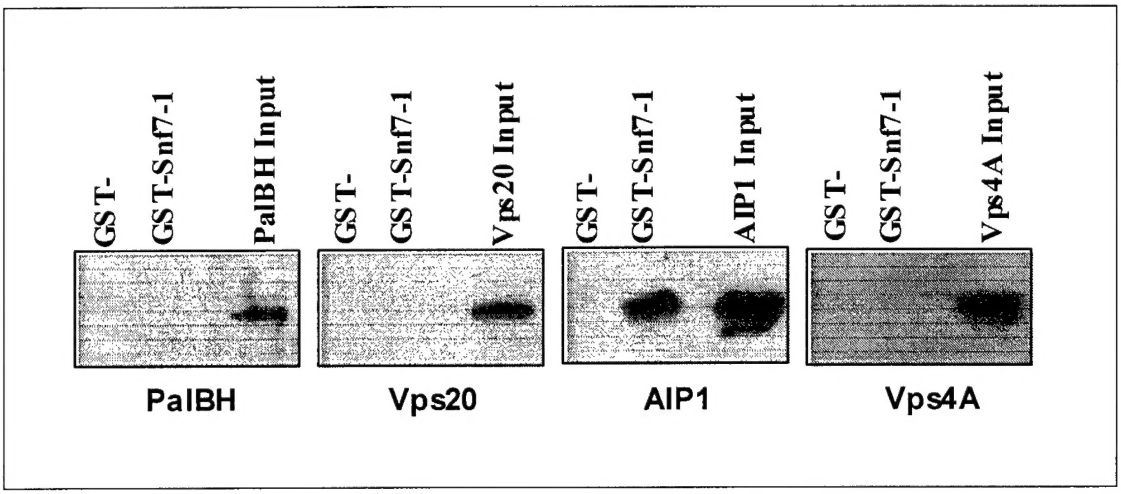

Figure 2 GST-capture experiments reveal that the Snf7-1-AIP1 interaction is functionally conserved with the yeast Snf7p-Bro1 and Snf7p-Rim20 interactions. Recombinant human GST-Snf7-1 was used to screen a several Epitope-tagged proteins expressed in Cos1 cells including: Vps family members (Vps20 and Vps4A), a Bro1 containing protein (AIP1), and an atypical calpain protease (PalBH) to identify potential binding partners. Of all proteins tested only AIP1 interacted with GST-Snf7-1.

The Interaction of hSnf-1 with AIP1 Requires the N-terminus of AIP1

In order to map the interaction between hSnf7-1 and AIP1, we attempted to generate several additional AIP1 constructs including AIP1-N-Δ1; amino acid residues 1-445, AIP1-N-Δ2; amino acids residues 1-208, AIP1-N-Δ3; amino acids residues 182-445, and AIP1-C-Δ1; amino acid residues 436-869 and tested their ability to bind GST-Snf7-1. We were only able to successfully express AIP1-N-Δ1 and AIP1-C-Δ1 in mammalian cells (Fig. 3). Using these two AIP1 mutants in affinity capture experiments with immobilized GST-Snf7-1 revealed that the N-terminus of AIP1 was both necessary and sufficient for interacting with Snf7-1 (Fig. 3). In contrast, the C-terminus of AIP1 was unable to bind hSnf7-1 (Fig. 3). In light of the fact that the region of AIP1 that interacts with Snf7 contains a Bro1 domain, we also tested whether the Bro1-containing protein Rhophilin-2 could interact with GST-hSnf7-1. While Rhophilin-2 was highly expressed, no interaction with GST-hSnf7-1 was detected (Fig. 3).

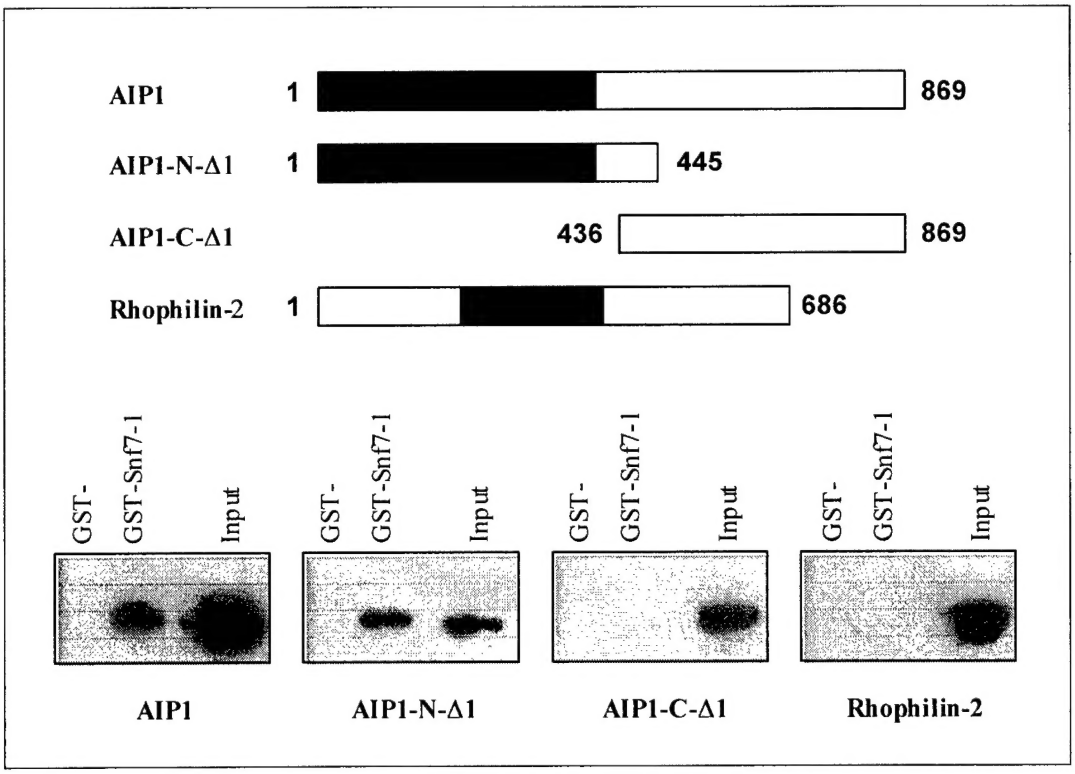

Figure 3 The interaction of AIP1 with Snf7-1 requires the N-terminus of AIP1
Schematic representation of the AIP1 protein and mutants expressed as FLAG-tagged proteins in Cos1 cells and used in GST-capture experiments. Respective positions in the proteins are indicated. The *stippled box* indicates the position of the Bro1 domain, the *black box* indicates a region conserved with Rim20/PalA homologs. The N-terminus, containing the Bro1 domain, of AIP1 interacts with Snf7-1 *in vitro*. FLAG-tagged AIP1-N-Δ1 and AIP1-C-Δ1 were expressed in Cos1 cells overnight and subject to in vitro binding with either GST- alone or GST-Snf7-1. Only the N-terminus of AIP1 (AIP1-N-Δ1) was able to interact with GST-Snf7-1.

III. KEY RESEARCH ACCOMPLISHMENTS & REPORTABLE OUTCOMES

- Attended the 2002 ASCB Conference in San Fransico CA. and presented the poster "The RhoA effector protein Rhophilin-2 interacts with α-actinin"
- Published: Peck, J. W., Oberst, M., Bouker, K. B., Bowden, E. & Burbelo, P. D. The RhoA-binding protein, Rhophilin-2, regulates actin cytoskeleton organization. *J Biol Chem* (2002), 277:43924-32.
- Received broad training in key microbiological techniques including: molecular cloning, tissue culture, and recombinant protein production.

IV. CONCLUSIONS

Here we have identified and cloned several members of the mammalian family Vps proteins including: hSnf7-1, AIP1, and hVps20. Using affinity-capture techniques, we identified AIP1 as an interacting partner of hSnf7-1. Additionally we mapped this

interaction to the N-terminus of AIP1 which contains a Bro1 domain. The interaction between hSnf7-1 and AIP1 is consistent with interactions observed in yeast, suggesting the possibility of additional Vps interactions are conserved from yeast to humans. These results suggest the possibility that the interaction of human Snf7-1 is likely to be biologically significant and may be involved in regulating MVB function. Future studies are aimed at further exploring the biological significance of the AIP1-Snf7-1 interaction with regards to receptor trafficking and degradation. Additionally we aim to identify and characterize the functional significance of other possible Vps interactions that occur in the mammalian system.

V. REFERENCES

1. Katzmann, D. J., Odorizzi, G. & Emr, S. D. Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol* **3**, 893-905 (2002).
2. Raymond, C. K., Howald-Stevenson, I., Vater, C. A. & Stevens, T. H. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. *Mol Biol Cell* **3**, 1389-402 (1992).
3. Babst, M., Katzmann, D. J., Snyder, W. B., Wendland, B. & Emr, S. D. Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. *Dev Cell* **3**, 283-9 (2002).
4. Babst, M., Katzmann, D. J., Estepa-Sabal, E. J., Meerloo, T. & Emr, S. D. Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. *Dev Cell* **3**, 271-82 (2002).
5. Tu, J., Vallier, L. G. & Carlson, M. Molecular and genetic analysis of the SNF7 gene in Saccharomyces cerevisiae. *Genetics* **135**, 17-23 (1993).
6. Kranz, A., Kinner, A. & Kolling, R. A family of small coiled-coil-forming proteins functioning at the late endosome in yeast. *Mol Biol Cell* **12**, 711-23 (2001).
7. Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G. & Superti-Furga, G. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **415**, 141-7 (2002).
8. Odorizzi, G., Babst, M. & Emr, S. D. Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. *Cell* **95**, 847-58 (1998).
9. Springael, J. Y., Nikko, E., Andre, B. & Marini, A. M. Yeast Npi3/Bro1 is involved in ubiquitin-dependent control of permease trafficking. *FEBS Lett* **517**, 103-9 (2002).
10. Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M. & Sakaki, Y. A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc Natl Acad Sci U S A* **98**, 4569-74 (2001).

11. Xu, W. & Mitchell, A. P. Yeast PalA/AIP1/Alix homolog Rim20p associates with a PEST-like region and is required for its proteolytic cleavage. *J Bacteriol* **183**, 6917-23 (2001).
12. Negrete-Urtasun, S., Denison, S. H. & Arst, H. N., Jr. Characterization of the pH signal transduction pathway gene palA of Aspergillus nidulans and identification of possible homologs. *J Bacteriol* **179**, 1832-5 (1997).
13. Vincent, O., Rainbow, L., Tilburn, J., Arst Jr, H. N., Jr. & Penalva, M. A. YPXL/I Is a Protein Interaction Motif Recognized by Aspergillus PalA and Its Human Homologue, AIP1/Alix. *Mol Cell Biol* **23**, 1647-55 (2003).
14. Che, S., El-Hodiri, H. M., Wu, C. F., Nelman-Gonzalez, M., Weil, M. M., Etkin, L. D., Clark, R. B. & Kuang, J. Identification and cloning of xp95, a putative signal transduction protein in Xenopus oocytes. *J Biol Chem* **274**, 5522-31 (1999).
15. Vito, P., Pellegrini, L., Guiet, C. & D'Adamio, L. Cloning of AIP1, a novel protein that associates with the apoptosis-linked gene ALG-2 in a Ca2+-dependent reaction. *J Biol Chem* **274**, 1533-40 (1999).
16. Missotten, M., Nichols, A., Rieger, K. & Sadoul, R. Alix, a novel mouse protein undergoing calcium-dependent interaction with the apoptosis-linked-gene 2 (ALG-2) protein. *Cell Death Differ* **6**, 124-9 (1999).
17. Wu, Y., Pan, S., Che, S., He, G., Nelman-Gonzalez, M., Weil, M. M. & Kuang, J. Overexpression of Hp95 induces G1 phase arrest in confluent HeLa cells. *Differentiation* **67**, 139-53 (2001).
18. Chen, B., Borinstein, S. C., Gillis, J., Sykes, V. W. & Bogler, O. The glioma-associated protein SETA interacts with AIP1/Alix and ALG-2 and modulates apoptosis in astrocytes. *J Biol Chem* **275**, 19275-81 (2000).
19. Borinstein, S. C., Hyatt, M. A., Sykes, V. W., Straub, R. E., Lipkowitz, S., Boulter, J. & Bogler, O. SETA is a multifunctional adapter protein with three SH3 domains that binds Grb2, Cbl, and the novel SB1 proteins. *Cell Signal* **12**, 769-79 (2000).
20. Chatellard-Causse, C., Blot, B., Cristina, N., Torch, S., Missotten, M. & Sadoul, R. Alix (ALG-2-interacting protein X), a protein involved in apoptosis, binds to endophilins and induces cytoplasmic vacuolization. *J Biol Chem* **277**, 29108-15 (2002).
21. Odorizzi, G., Katzmann, D. J., Babst, M., Audhya, A. & Emr, S. D. Bro1 is an endosome-associated protein that functions in the MVB pathway in Saccharomyces cerevisiae. *J Cell Sci* **116**, 1893-903 (2003).
22. Babst, M., Wendland, B., Estepa, E. J. & Emr, S. D. The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. *Embo J* **17**, 2982-93 (1998).
23. Futai, E., Kubo, T., Sorimachi, H., Suzuki, K. & Maeda, T. Molecular cloning of PalBH, a mammalian homologue of the Aspergillus atypical calpain PalB. *Biochim Biophys Acta* **1517**, 316-9 (2001).